

# Agents That Increase the Permeability of the Outer Membrane

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## INTRODUCTION

Gram-negative bacteria are resistant to a large number of noxious agents as a result of the effective permeability barrier function of their outer membrane (OM) (for reviews, see references 57, 105, 123, and 131–138). The OM is impermeable to macromolecules and allows only limited diffusion of hydrophobic substances through its lipopolysaccharide (LPS)-covered surface. The outer leaflet of the enterobacterial OM lacks glycerophospholipids and, hence, the effective channels for hydrophobic diffusion (134, 136). The OM of these bacteria is also resistant to neutral and anionic detergents. Small hydrophilic compounds diffuse through the OM via the water-filled porin channels, but the narrowness of these channels remarkably restricts their diffusion (131, 133, 137). Since many of the harmful agents, including antibiotics, are either hydrophobic or relatively large hydrophilic compounds, they penetrate the OM poorly or rather poorly (130–137). Furthermore, the polysaccharide constituents of the OM help bacteria to evade phagocytosis and protect the deeper parts of the OM from complement and antibody binding.

The molecular basis of the integrity of the OM lies in its LPS (137, 138). LPS binds cations, since it is polyanionic because of a number of negative charges in its lipid A and inner-core parts. Adjacent polyanionic LPS molecules are apparently linked electrostatically by divalent cations ( $\text{Mg}^{2+}$ ,  $\text{Ca}^{2+}$ ), inherent in the OM, to each other to form a stable “tiled roof” on the surface of the OM (88, 134, 138).

Accordingly, the OM is a remarkable barrier and the cation-binding sites of LPS are essential for the integrity of the OM. However, these sites are, simultaneously, also the Achilles’ heel of the OM. It has long been known that the naturally occurring polycationic antibiotics of the polymyxin group complex avidly with LPS and disorganize the whole

OM (57, 137, 182). This is not their lethal action, but it is their means of permeating the OM to reach their final target, the cytoplasmic membrane. Also, the OM-disorganizing and -permeabilizing action of EDTA, which chelates  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ , is well known (57, 102, 105, 137). However, during the last few years, growing evidence that numerous other agents, most of them cationic, have notable OM-damaging action, has accumulated. Some of these agents are only weakly bactericidal but are remarkably effective in permeabilizing the OM to other agents. These permeabilizers are useful tools in various cellular studies in which the impermeability of the OM poses problems. They might also be useful in the design of such permeabilizer compounds, which could be used therapeutically to widen the spectrum of clinically used antibiotics.

Besides the compounds which have a strong OM-permeabilizing activity, there are others which might have a weak permeabilizing action. In addition, numerous new antibacterial cationic peptides from various vertebrate and invertebrate host tissues have been discovered, and others have been characterized in detail. These cationic peptides are effective against gram-negative bacteria, apparently penetrate through the OM by interacting with LPS, and are believed to play an important role in the host defense against invaders.

This review critically summarizes the recent data on the agents which permeabilize the OM, or penetrate through the OM, by interacting with LPS. The review is also a short synopsis on gram-negative bacteria and mutants which are resistant to such agents as a result of having an unusual or altered OM.

The review excludes the description of antimetabolites and inhibitors such as diazaborine (91, 193) and ketodeoxyoctulosonate analogs (52, 53) which increase the permeability of the OM by disturbing or inhibiting the biosynthesis of

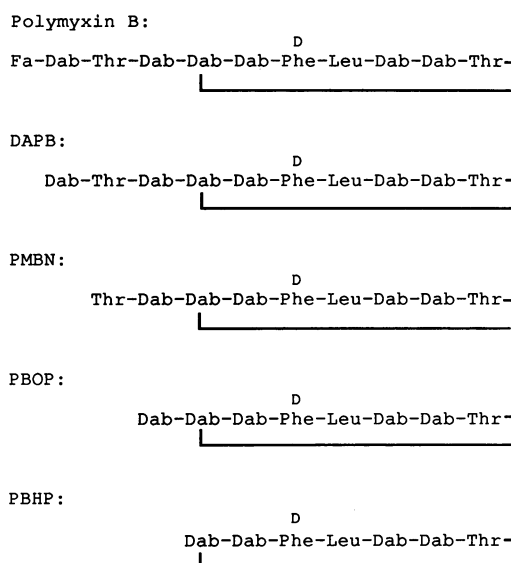


FIG. 1. Structure of polymyxin B and its derivatives deacylpolymyxin B (DAPB), PMBN, polymyxin B octapeptide (PBOP), and polymyxin B heptapeptide (PBHP). Abbreviations: Dab, diamino-butyric acid; Fa, fatty acid (6-methyl-octanoic acid or 6-methyl-heptanoic acid).

its components. Also excluded is OM permeabilization by physical damage such as that caused by heat (191, 192), UV irradiation (117), drying (154), or freeze-thawing (11).

### CATIONIC AGENTS AS OM PERMEABILIZERS

#### Polymyxin, Polymyxin Nonapeptides, and Other Derivatives

Polymyxins are pentacationic amphipathic lipopeptide antibiotics characterized by a heptapeptide ring and a fatty acid tail (182). They are bactericidal to gram-negative bacteria by a dual mechanism of action. Polymyxin first binds to the OM and permeabilizes it (the sublethal action). This allows it to enter the cytoplasmic membrane, where it causes leakage of cytoplasmic components (the lethal action). The lethal action takes place at almost the same polymyxin concentration as that required for the OM-permeabilizing action, but it is still possible to separate the effects (157, 182, 183).

Polymyxin derivatives which lack the fatty acid tail (Fig. 1) are less bactericidal or not bactericidal at all (25, 182) but, as first shown by Vaara and coworkers (198, 209–211, 221), have preserved a notable OM-permeabilizing action (Table 1; see also the references therein). In this respect, the best-characterized derivative is polymyxin B nonapeptide (PMBN). Its MICs against *Escherichia coli* and *Salmonella typhimurium* are  $\geq 300$   $\mu\text{g/ml}$ , but even as low a concentration as 0.3 to 1  $\mu\text{g/ml}$  is sufficient to permeabilize the OM, as evidenced by a drastic sensitization to the hydrophobic antibiotics against which the intact OM is an effective barrier. PMBN also sensitizes *E. coli* and *S. typhimurium* to the bactericidal activity of serum complement (90, 113, 217, 218). Also, polymyxin decapeptides (deacylpolymyxins) (220), colistin nonapeptide (76), polymyxin B octapeptide (85), and polymyxin B heptapeptide (85), which all still carry the cyclic heptapeptide, are effective permeabilizers of the OM, whereas polymyxinlike synthetic compounds in which the heptapeptide is not cyclic (202) lack the activity (Table 1). This indicates that the cationicity alone is not the sole

TABLE 1. Growth-inhibiting and OM permeability-increasing properties of polymyxin derivatives and lysine polymers in *E. coli* and *S. typhimurium*

Polycation	MIC ( $\mu\text{g/ml}$ ) <sup>a</sup>	OM-permeabilizing concn ( $\mu\text{g/ml}$ ) <sup>b</sup>	Reference(s)
Polymyxin B	1	0.3–1	210
DAPB	10–30	0.3–1	85, 220
Deacylcolistin	30	1–3	220
PMBN	$\geq 300$	0.3–1	85, 209, 210, 220, 221
Colistin nonapeptide	$\geq 100$	1	76, 205
Polymyxin B octapeptide	$\geq 300$	1–3	85
Polymyxin B heptapeptide	$\geq 300$	1–3	85
Linear lysyl PMBN <sup>c</sup>	$\geq 300$	$\geq 300$	202
Linear arginyl DAPB <sup>d</sup>	$\geq 300$	$\geq 300$	202
Tetralysine	$\geq 100$	$\geq 100$	210
Pentalysine	$\geq 100$	$\geq 100$	205
Lysine <sub>20</sub>	30–100	0.3–3	210, 214
Lysine <sub>50</sub>	3	1	210

<sup>a</sup> The minimal concentration of the polycation required to inhibit visual bacterial growth in L broth (221) (polymyxin B derivatives) or in D minimal medium (210) (colistin nonapeptide and lysine polymers) in a standard 18-h assay.

<sup>b</sup> The minimal concentration of the polycation required to decrease the MICs of a set of hydrophobic antibiotics by a factor of  $\geq 10$ . Assay conditions were identical to those described in footnote a.

<sup>c</sup> Sequence: Thr-Lys-Lys-Lys-DPhe-Leu-Lys-Lys-Thr.

<sup>d</sup> Sequence: Arg-Thr-Arg-Arg-Arg-DPhe-Leu-Arg-Arg-Thr.

determinant required for the OM-permeabilizing activity and that a proper conformation is crucial.

Polymyxin nonapeptides bind to LPS (86, 148, 197, 215) and detoxify its endotoxicity (33, 227). However, the affinity of PMBN to isolated LPS ( $K_d$ , 1.5  $\mu\text{M}$ ), isolated OM ( $K_d$ , 1.1  $\mu\text{M}$ ), and native bacterial cell surface ( $K_d$ , 1.3  $\mu\text{M}$ ) is clearly (ca. 3- to 10-fold) lower than that of polymyxin (162, 205, 215). PMBN is rather weak in replacing the hydrophobic fluorescent probe, dansyl polymyxin, from its binding sites in the LPS of *Pseudomonas aeruginosa* (118) but clearly more potent when competing with natural polymyxin B for binding to the enterobacterial OM (215). *E. coli* and *S. typhimurium* bind approximately  $1 \times 10^6$  to  $2 \times 10^6$  molecules of PMBN per cell (215), which corresponds to approximately 0.5 to 1 mol of PMBN per mol of LPS.

The molecular mechanism by which PMBN increases the permeability of OM has not been elucidated, but it is not due to any marked LPS release or the activation of the OM-associated phospholipase (detergent-resistant phospholipase A<sub>1</sub>) (211, 214). Interestingly, PMBN expands the outer leaflet of the OM so that fingerlike projections involving only this leaflet can be found in electron-microscopic thin sections (211). Those projections are seen also in polymyxin B-treated cells. In addition, PMBN dramatically increases the cell surface hydrophobicity of *E. coli*, as measured by partitioning of cells between buffer and *p*-xylene (24), and enhances the partitioning of a hydrophobic probe, *N*-phenyl naphthylamine (NPN) to isolated LPS (205, 207). This could suggest that the permeabilizing effect of PMBN may ultimately be explained by the alteration of the physical structure of the OM bilayer. Further experiments in this direction are crucial to our understanding of this process. As will be discussed later in this review (in the section on molecular mechanisms of the OM permeability increase), the most promising approaches would undoubtedly be (i) studies which measure the supramolecular organization of isolated

TABLE 2. PMBN-induced sensitivity increase to antibiotics and other drugs in *E. coli* and *S. typhimurium*

Drug	Sensitivity increase <sup>a</sup>	Reference(s)
Rifampin	30–300 (30)	76, 85, 199, 209, 210, 220, 221
Rifabutin	30–100	205
Fusidic acid	30–300	36, 85, 87, 113, 199, 209, 210, 220, 221
A23187	≥300	3
Vancomycin	260 <sup>b</sup>	90
Mupirocin	100	203
Valinomycin	50 <sup>c</sup>	3
Novobiocin	10–100 (8)	76, 85, 113, 199, 209, 210, 220, 221
Erythromycin	8–30 (30)	76, 85, 87, 113, 176, 199, 209, 210, 220, 221
Clindamycin	10–30	85, 199, 209, 210, 220, 221
Azithromycin	10–30	204
Roxithromycin	10	204
Clarithromycin	10	204
Nafcillin	10–30	199, 210, 221
Cloxacillin	5–30	85, 113, 199, 209, 210
Nalidixic acid	8–10	85, 199
Lincomycin	2–8	85
Carbenicillin	3	221
Benzylpenicillin	1–3	209, 210, 221
Cefuroxime	2	199
Ampicillin	1	221
Chlortetracycline	1	221
Tetracycline	1	221
Ciprofloxacin	1	85
Norfloxacin	1	85
Ofloxacin	1	85

<sup>a</sup> The approximate ratio of the MIC of the drug in the absence of PMBN to that obtained in the presence of PMBN (3 µg/ml unless otherwise stated). Values in parentheses are the corresponding sensitivity increase ratios related to colistin nonapeptide (2.5 µg/ml).

<sup>b</sup> At a PMBN concentration of 10 µg/ml.

<sup>c</sup> At a PMBN concentration of 5 µg/ml.

LPS in the presence of PMBN and (ii) studies which involve artificial asymmetric planar LPS-glycerophospholipid bilayers.

Notably, PMBN increases predominantly the penetration of hydrophobic antibiotics. A PMBN concentration as low as 3 µg/ml decreases their MICs against *E. coli* and *S. typhimurium* by a factor of 10 to 300 (Table 2). The resultant

very low MICs are comparable to those determined for such enterobacterial strains as the *acrA* (29), DC2 (28), *ssc* (68), *envA* (239), *lpxA* (224), and SS-B (199) strains and the deep rough heptoseless strains (156, 160, 185), all of which have a mutational defect in the OM permeability barrier (Table 3). This PMBN-induced sensitivity increase has been documented in multiple studies involving various assay broths (see the references in Table 2), and it can be demonstrated also with antibiotic disks on PMBN-containing Iso-Sensitest agar (216). Because the action of many cationic permeabilizers is reversed by physiological concentrations of NaCl and divalent cations (see below), it should be noted here that PMBN has a very powerful action in Luria broth (nearly 0.1 M NaCl [85, 199, 202, 220, 221], in Medium B of reference 218 (150 mM NaCl), in D minimal broth (0.4 mM Mg<sup>2+</sup> [113, 197, 209–211]) and in Trypticase soy broth (1 mM Mg<sup>2+</sup> [90]). Because of this potent OM-permeabilizing ability, PMBN and polylysines (see below) have been exploited as useful tools in various cellular studies (2, 44, 83, 150, 237).

PMBN has only a slight, if any, effect on the MICs of antibiotics which are believed to traverse the OM through porin pores. The PMBN-induced permeabilization does not include the release of periplasmic proteins (211). To release them, a very high PMBN concentration (200 µg/ml) in an unphysiological buffer is required (36). These conditions also release cytoplasmic components (37).

Besides enteric bacteria, *P. aeruginosa* is also very sensitive to the OM-permeabilizing action of PMBN (87, 201, 218, 219, 221). Polymyxin-resistant bacteria such as *Proteus* spp. and the *pmrA* mutants of *S. typhimurium* bind less PMBN (215) and are resistant to its action (197, 218, 221). Notably, they are also resistant to numerous other cationic agents (see below). Other polymyxin- and PMBN-resistant gram-negative bacteria include neisseriae and *Serratia marcescens* (190, 218, 221).

Like many other strongly cationic substances such as defensins (7) (see below), PMBN rapidly and unselectively binds to tissues and has a short half-life in serum (25 min) (129). Limited studies on the protective ability of PMBN (with or without a hydrophobic antibiotic) in experimental gram-negative infection models have been disappointing (90). Regarding toxicity, in effective-dose comparisons, polymyxin nonapeptide was 15 times less toxic than polymyxin in an acute-toxicity assay in mice (25), 25 times less active in releasing histamine from rat mast cells (222),

TABLE 3. Comparison of the PMBN-induced and mutational sensitization of *E. coli* and *S. typhimurium* to hydrophobic antibiotics

Permeabilized or mutant bacteria			Control bacterium	Sensitization factor <sup>a</sup> to:					Reference(s)
Strain	Permeabilizer or mutation	Species		Erythromycin	Clindamycin	Fusidic acid	Novobiocin	Rifampin	
IH3080	PMBN (3 µg/ml)	<i>E. coli</i>	IH3080, no PMBN	10	≥10	30	30	30	221
IH3080	PMBN (10 µg/ml)	<i>E. coli</i>	IH3080, no PMBN	30	≥10	100	100	100	221
SH5014	PMBN (3 µg/ml)	<i>S. typhimurium</i>	SH5014, no PMBN	30	≥30	300	100	300	199
SM101	<i>lpxA2</i>	<i>E. coli</i>	SM105 ( <i>lpxA</i> <sup>+</sup> )	64	32	≥128	64	64	224
D21f2	<i>rfa</i> <sup>b</sup>	<i>E. coli</i>	SM105 ( <i>rfa</i> <sup>+</sup> )	32	4	≥64	64	64	224
CL94	<i>acrA</i>	<i>E. coli</i>	CL93 ( <i>acrA</i> <sup>+</sup> )	3	ND <sup>c</sup>	<2	>80	3	29, 184
DC2	<i>abs</i>	<i>E. coli</i>	UB1005 ( <i>abs</i> <sup>+</sup> )	4	ND	ND	40	4	28
LS583	<i>envA1</i>	<i>E. coli</i>	LS584 ( <i>envA</i> <sup>+</sup> )	>64	ND	>32	4	64	239
SL1102	<i>rfaE</i> <sup>b</sup>	<i>S. typhimurium</i>	SH5014 ( <i>rfaE</i> <sup>+</sup> )	≥30	30	30	≥30	30	205, 210
SH7616	SS-B	<i>S. typhimurium</i>	SH5014 (SS-B <sup>+</sup> )	32	32	≥128	128	2	199
SH7622	<i>ssc-1</i>	<i>S. typhimurium</i>	SH5014 ( <i>ssc</i> <sup>+</sup> )	64	32	≥256	ND	≥135	68

<sup>a</sup> The approximate ratio between the MIC for control bacteria and that for the corresponding PMBN-permeabilized or mutant bacteria.

<sup>b</sup> Encodes the deep rough LPS chemotype Re.

<sup>c</sup> ND, not determined.

approximately 100 times less toxic in a eucaryotic cytotoxicity assay (39), and approximately 150 times less active in causing neuromuscular blockade (107). Also, the comparisons of Danner et al. (33) were favorable to PMBN. However, it was realized that the nephrotoxicities (proximal tubular injury in young male rats) of PMBN and polymyxin B were almost identical (129). Therefore, PMBN can be expected to be too toxic to be used clinically. The mechanism of nephrotoxicity of strongly cationic peptides might resemble that of aminoglycosides, which have high affinity to the anionic phospholipids (particularly phosphatidylinositol) of the kidney brush border membrane (149, 236).

### Lysine Polymers and Protamine

Pentalysine (five net positive charges) is a very weak permeabilizer of the OM (201). It is inactive against *E. coli* and *S. typhimurium* but has some OM-permeabilizing activity against *P. aeruginosa*, provided that the assay is performed at low ionic strength, at which the binding of pentalysine to the OM is less inhibited by competing monovalent buffer cations. Such buffer cations have previously been shown to inhibit a weak action of polymyxin against the polymyxin-resistant *pmrA* strains of *S. typhimurium* (195). Tri- and tetralysine lack any permeabilizing activity (201, 210). As first shown by Vaara et al. (196, 210, 214), the longer lysine polymer, lysine<sub>20</sub>, is an effective permeabilizer (Table 1). A concentration of lysine<sub>20</sub> as low as 0.3 to 3 µg/ml is sufficient to sensitize *E. coli* and *S. typhimurium* to hydrophobic antibiotics by a factor of 10 to 100, whereas the MIC of lysine<sub>20</sub> against these bacteria is 30 to 100 µg/ml. Longer polymers such as lysine<sub>50</sub>, as well as the strongly cationic protamine from salmon sperm (32 amino acid residues, 21 net positive charges), resemble polymyxin in being both permeabilizers and effective bactericidal agents (83, 210).

Like PMBN, lysine<sub>20</sub> permeabilizes the OM to hydrophobic compounds (210, 211). However, in contrast to PMBN, it releases a marked proportion (30 to 40%) of LPS from the cells (73, 211) and, accordingly, could create compensatory phospholipid bilayer regions in the OM. These regions could act as hydrophobic diffusion channels. Also, protamine releases LPS (up to approximately 40% of the total) (73, 211). At high concentrations, both lysine<sub>20</sub> and protamine liberate periplasmic proteins (211). Furthermore, because of the high affinity of protamine to LPS, protamine-agarose removes LPS from solution with an efficiency of 99.5 to 99.9% (67).

Besides polylysines, polyornithine is also a potent permeabilizer of the OM (64). ε-Polylysines with more than 10 residues are effective bactericidal agents (175); it would be interesting to measure the OM-permeabilizing action of the shorter ε-polylysines. Polymers of basic amino acids also include those present in nourseothricin. The antibiotic nourseothricin is a mixture of streptothricins, which consist of a nucleoside part and an ε-poly-β-lysine part (1 to 6 residues in streptothricins A to F, respectively). High concentrations (20 to 50 µg/ml) of nourseothricin permeabilize the OM of *E. coli* to the anionic detergent deoxycholate and increase the bacterial susceptibility to lysis by lysozyme (168), as do polymyxin, polylysines, and protamine (196, 205, 208, 211).

### Other Small Polycationic Peptides

Defensins (29 to 34 residues, 3 to 10 net positive charges), cecropins (31 to 39 residues, 4 to 8 net positive charges), magainins (23 residues, 3 to 4 net positive charges), and melittin (26 residues, 6 net positive charges) are cationic oligopeptides present in mammalian phagocytic cells, insect hemolymph, frog skin, and bee venom, respectively (for recent reviews, see references 13–15, 96, and 97). All form channels in artificial membranes; cecropins, magainins, and melittin do so by virtue of their suitable α-helical structure (27, 38, 79, 189). All are toxic to a wide variety of target cells including gram-positive and -negative bacteria, yeasts (defensins and magainins), blood cells (melittin), *Plasmodium falciparum* (magainin, cecropin B, cecropin-melittin hybrids), mammalian tumor cells (magainin, defensins), and enveloped viruses (defensins) (14–16, 32, 96, 158, 225, 240, 241). Defensins differ from the others in killing only actively metabolizing microbes (50, 94, 96). Furthermore, their activity is inhibited by monovalent (50 to 70 mM Na<sup>+</sup>) cations (55, 98, 167). Insect defensins (92), defensin-resembling crab hemocyte peptides (116, 124), and a mammalian cecropinlike intestinal substance (93) are also known.

In analogy to polymyxin, defensins, cecropins, magainins, and melittin must cross the OM to reach their final target, the cytoplasmic membrane. As cationic substances, they can be expected to bind to LPS; this binding has been demonstrated with defensin (161) and magainin (153). Furthermore, assays measuring the crypticity of periplasmic β-lactamase or the uptake of NPN have indicated that a lethal defensin concentration increases the permeability of the OM (94, 161). However, the highest concentrations of human defensins which still permit bacterial growth (3 µg/ml in low-ionic-strength medium, 100 µg/ml in a medium with normal ionic strength) were unable to permeabilize the OM of *E. coli* and *P. aeruginosa* to hydrophobic probe antibiotics (219). Accordingly, the OM permeability-increasing action of defensins can be regarded as very weak, compared with that of polymyxins. Certain synthetic magainin derivatives have been described to have synergism with the hydrophobic antibiotic erythromycin (106); details of this study have not yet been published. No results of studies on the effect of subinhibitory concentrations of melittin and cecropins on the function of the OM are available.

Other cationic antibacterial oligopeptides include bactericins of bovine neutrophils, seminalplasmin of bull sperm, and apidaecins and abaecins of bee lymph. Bactericins Bac5 (42 residues, 10 net positive charges) and Bac7 (42 residues, 10 net positive charges) are arginine and proline rich (47), are active only against gram-negative bacteria (MIC for *E. coli*, 12 to 50 µg/ml) (51, 179), and, at bactericidal concentrations, increase the permeability of the OM (assayed by measuring the crypticity of β-lactamase) (179). Seminalplasmin (48 residues, 9 net positive charges) lyses gram-positive and -negative bacteria as well as dividing eucaryotic cells (26, 178); its OM-permeabilizing properties have not yet been studied. Even less is known about apidaecins (18 residues, 4 net positive charges; they kill exclusively gram-negative bacteria [22]) and abaecins (which have a wider inhibitory spectrum [23]).

The cationic lanthionine antibiotic nisin (30 residues, 3 lysines), produced by *Lactococcus lactis* and active against other gram-positive bacteria (158), does not increase the OM permeability to hydrophobic antibiotics (71, 205).

### Bactericidal/Permeability-Increasing Protein

The bactericidal/permeability-increasing protein (BPI), discovered and extensively studied by Weiss and Elsbach and their collaborators, is an approximately 58-kDa strongly cationic protein present in the azurophil granules of human and rabbit neutrophils. The sequence of BPI has been determined (54). Two neutrophil granule proteins isolated in other laboratories, CAP57 (171) and BP55 (72), appear to be identical with BPI (96, 146, 180, 228). BPI is inactive against gram-positive bacteria but is an effective bactericidal agent against gram-negative bacteria (MIC, as low as 0.5 to 1  $\mu\text{g/ml}$ ) (42, 145, 171, 231). It has strong affinity to LPS, inactivates its endotoxicity (111, 144), and binds with an apparent  $K_d$  of 23 nM to *E. coli* cells ( $2.2 \times 10^6$  binding sites per cell) (109). Accordingly, the affinity of BPI is remarkably higher than that of PMBN or polymyxin B (see above), but the number of binding sites is comparable. Polymyxin-resistant enteric bacteria (the *pmrA* mutants of *S. typhimurium*, *Proteus* spp., and *Serratia marcescens*) are resistant to BPI (9, 45, 72, 171, 180, 181, 232). The bactericidal activity resides within the N-terminal 25-kDa fragment of BPI, which has a net positive charge of 16 (145). Lethal concentrations of BPI and its 25-kDa fragment increase the OM permeability to a hydrophobic probe antibiotic, actinomycin D (42, 145, 232), but as with polymyxin, it is possible to separate the lethal and sublethal effect by using suitable procedures (for BPI, e.g., restoration of the OM function by 80 mM  $\text{Mg}^{2+}$  or rescue of viability by serum albumin) (110, 233, 234).

BPI has remarkable sequence homology to the LPS-binding protein produced by hepatocytes and appearing as a serum acute-phase reactant (165, 187). This homology extends through the entire sequence. LPS-binding protein has very high affinity to LPS ( $K_d$ , ca. 1 nM [188]) but lacks the bactericidal activity (187). It would be interesting to measure the effect of LPS-binding protein on the OM permeability barrier function.

### Other Large Cationic Peptides

One group of cationic leukocyte proteins are those located in the granules of eosinophils. Both the eosinophil major basic protein and the eosinophil cationic protein (ECP) are antibacterial against *E. coli* and *Staphylococcus aureus*; lethal concentrations of these proteins permeabilize the OM, as evidenced by measuring the crypticity of the periplasmic  $\beta$ -lactamase (99).

Lactoferrin is a 78-kDa, slightly basic (pI, 8.7) glycoprotein which inhibits microbial growth by sequestering  $\text{Fe}^{3+}$ . High concentrations (125 to 2,000  $\mu\text{g/ml}$ ) of unsaturated lactoferrin (i.e., apolactoferrin), but not lactoferrin saturated with  $\text{Fe}^{3+}$ , release LPS from *E. coli* and *S. typhimurium* in suitable buffers; 2,000  $\mu\text{g}$  of apolactoferrin per ml sensitizes *E. coli* in growth medium to a hydrophobic probe, rifampin (40, 41). The mechanism of apolactoferrin-induced release of LPS has not been studied, but it has been suggested (41) that a high concentration of apolactoferrin could remove stabilizing divalent or other cations from the OM as does EDTA (see below).

Azurocidin and cathepsin G are very cationic proteins present in neutrophil granules. They share strong sequence homology with each other and with the serine protease neutrophil elastase, which is present in the same granules (4). Azurocidin (sequence identical with that of CAP37 [151]) lacks proteolytic activity (19) and is active against polymyx-

in-susceptible gram-negative strains (19, 48, 96, 171, 172); however, BPI is clearly more potent on molar basis (48, 171). Azurocidin is also active against *Enterococcus faecalis* and *Candida albicans* (48). Cathepsin G has proteolytic chymotrypsinlike activity. It is inhibitory against bacteria, especially *Neisseria gonorrhoeae*, and against *C. albicans*; the antimicrobial mechanism appears to be independent of its enzymatic activity (96, 139, 173). It also appears to be independent of the polycationicity of the protein, since cathepsin-derived peptides IIGGR and HPQYNQR are bactericidal against *Neisseria gonorrhoeae* and *Staphylococcus aureus* (8, 174). The optimal microbicidal activity of both azurocidin and cathepsin G requires a low-ionic-strength medium (96, 139, 172), as does that of defensins. The effect of azurocidin and cathepsin G on the OM permeability barrier has not been studied. Neutrophil elastase is also strongly cationic. Besides being proteolytic, it has been shown to potentiate the bactericidal activity of cathepsin G by a nonenzymatic activity (140).

The anti-LPS factor of the horseshoe crab *Tachypleus tridentatus* is a basic polypeptide (102 residues) which inhibits the LPS-mediated crab hemolymph coagulation system and binds to LPS (1, 121). Since it lyses LPS-coated erythrocytes (141), as does polymyxin (20), one could suggest that it damages the bacterial OM as well. The factor has bactericidal activity against enterobacterial mutants which produce defective LPS (MIC, ca. 0.1 to 1  $\mu\text{g/ml}$  [121]); its effect on the OM permeability barrier has not been studied. The sequence of the analogous anti-LPS factor from *Limulus polyphemus* is also known (122).

Attacins ( $M_w$ , 20 kDa) from the hemolymph of the cecropia moth (15, 74) and the partially homologous sarcotoxin IIA from the flesh fly (5, 6) are inhibitory against *E. coli* and certain other gram-negative bacteria (MIC, ca. 20 to 160  $\mu\text{g/ml}$ ). Sarcotoxin IIA and one of the two attacins are cationic. Attacins potentiate (additively or synergistically) the antibacterial activity of cecropins (see above) against *E. coli* (15, 43), and a bactericidal concentration of basic attacin sensitizes the cell to Triton X-100 (43). Recently, it has been shown that neutral attacin at 400  $\mu\text{g/ml}$  reduces the synthesis of major OM proteins in pulse-labeling (21). On the basis of these results, it has been claimed that the antibacterial action of attacins is specifically directed against the OM and that the other effects of attacins are indirect consequences of this effect (21, 43). However, more experimental data are needed before such conclusions can be made.

### Compound 48/80

Compound 48/80 is a polycationic polymer of an organic monovalent cation, *p*-methoxyphenethylmethylamine. It has essentially no surfactant activity and is a relatively weak antimicrobial agent against bacteria, fungi, and protozoa (56, 103). Like polymyxin (222), defensins (238), and magainins (70), it effectively liberates histamine from mast cells by a selective, noncytolytic action (56, 103). Katsu et al. showed that compound 48/80 permeabilizes the OM of *E. coli* to a hydrophobic probe, tetraphenylphosphonium ion, as do polymyxin and lysine<sub>20</sub> (83, 84). This permeabilization was evident at concentrations (2 to 5  $\mu\text{g/ml}$ ) of compound 48/80 clearly lower than those which were toxic to the cytoplasmic membrane. Furthermore, they showed that their "48/80 dication" preparation (which they presume to consist of dimeric *p*-methoxyphenethylmethylamine only) was more than 10 times less active as an antibacterial agent than the

polymer (81, 82). It was also less active as an OM permeabilizer, even though it retained some activity (82).

### Aminoglycosides

Aminoglycosides (three to six net positive charges) bind to isolated LPS (34, 61, 118, 148) and have a weak OM-permeabilizing action. As shown by Hancock and collaborators, they permeabilize the OM of *P. aeruginosa* to the hydrophobic probes NPN and nitrocefin (63, 64, 104). This action was demonstrated by using 5 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer. The presence of 50  $\mu$ M  $Mg^{2+}$  reduced the effect of gentamicin (20  $\mu$ g/ml) by 90% (104); in the presence of 1 mM  $Mg^{2+}$ , no permeabilizing action was found (63, 64). Accordingly, the aminoglycoside effect is reminiscent of that of pentamidine (see above), which is very sensitive to inhibition by  $Mg^{2+}$  as well as monovalent cations (150 mM  $Na^+$ ). However, in a direct comparison, pentamidine is more potent than gentamicin (201). Also, the OM of enteric bacteria can be permeabilized in 5 mM HEPES by aminoglycosides (61), but, again, the aminoglycoside effect must be regarded as weak since in growth medium, streptomycin (tested up to 100  $\mu$ g/ml) failed to sensitize *S. typhimurium* (*rpsL*) to a hydrophobic probe (novobiocin) whereas both PMBN and lysine<sub>20</sub> sensitized it by a factor of 10 to 30 at 0.3  $\mu$ g/ml and by a factor of 30 to 100 at 1  $\mu$ g/ml (210).

Accordingly, aminoglycosides are only very weak permeabilizers. Whether they could act synergistically with other permeabilizers, such as those produced by leukocytes, has not yet been studied.

### Tris, $Ca^{2+}$ , $Mg^{2+}$ , and $Na^+$

Tris is a bulky primary amine and, at high concentrations, an OM permeability-increasing agent. A solution containing 35 mM Tris (pH 7.4) permeabilizes the OM of *P. aeruginosa* to nitrocefin (63); 200 mM Tris sensitizes this bacterium to lysozyme (63). The bactericidal effect of deoxycholate and dodecyl sulfate on *S. typhimurium* is potentiated by 50 mM Tris (pH 7.2), as well as by high concentrations (250 to 500 mM) of NaCl (205); 100 mM Tris renders the OM of an LPS-defective Rc mutant susceptible to labeling with dansyl chloride (163). Tris and EDTA work synergistically in releasing LPS from enteric bacteria (see below); 100 mM Tris (pH 7.2) alone, without EDTA, releases 20% of the LPS of smooth *S. typhimurium* (205). A significant proportion (21 to 42%) of the periplasmic  $\beta$ -lactamase of *S. typhimurium* can be released by treating the cells in the cold with 50 to 90 mM Tris (pH 7.2) or 200 to 300 mM NaCl (196).

It can be suggested that Tris binds to LPS, replaces stabilizing  $Ca^{2+}$  and  $Mg^{2+}$ , and reduces the interaction between LPS molecules (137, 138), as does triethylamine, the known effective dispersant of isolated LPS (49). Also, ethanolamine and pyridine dissociate aggregates of isolated LPS (49); it can be expected that they and many other organic monovalent cations have an OM-permeabilizing effect similar to that of Tris (see also the synergistic action of EDTA and organic cations, below). The effect of excessive  $Na^+$  can also be expected to involve replacement of  $Ca^{2+}$  and  $Mg^{2+}$  from their binding sites in LPS (69).

The OM-permeabilizing effect of  $Ca^{2+}$  at 20 mM or more in the cold has been well described (18, 137). The most likely explanation is that the excessive binding of  $Ca^{2+}$  to LPS "freezes" the LPS monolayer by raising its melting temperature and that this easily creates OM cracks, through which

macromolecules can diffuse. Also, cold 100 mM  $Mg^{2+}$  permeabilizes the OM (196).

### Cationic Detergents

Gram-negative enteric bacteria are remarkably resistant to anionic and neutral detergents but sensitive to the monocationic detergents such as benzalkonium chloride (BAC) and cetyltrimethylammonium chloride. The OM-permeabilizing action of the cationic detergents is poorly characterized. A very high concentration of BAC (20 mg/ml) increases the uptake of NPN through the OM of *P. aeruginosa* (64), whereas the periplasmic  $\beta$ -lactamase can be released from *S. typhimurium* by a BAC concentration of 27  $\mu$ g/ml (196). Although the polymyxin-resistant *pmrA* mutants of *S. typhimurium* are cross-resistant to a wide variety of other cationic agents (see below), they are supersusceptible, when tested in the growth inhibition test, to both BAC and cetyltrimethylammonium chloride and are slightly sensitized to anionic detergents as well (196). Furthermore, although *Proteus mirabilis* is naturally resistant to polymyxin, its BAC-resistant mutants are polymyxin sensitive (205). Accordingly, the effect of BAC on the OM is probably very different from that of polymyxin; perhaps hydrophobic interactions play a larger role in that effect than ionic interactions.

### CHELATORS AS OM PERMEABILIZERS

As reviewed previously (57, 102, 105, 137), EDTA has a profound effect on the OM permeability barrier of gram-negative enteric bacteria and *P. aeruginosa*. It removes, by chelation, stabilizing divalent cations from their binding sites in LPS. This results in the release of a significant proportion of LPS from the cells, as first shown by Leive in 1965 (101). The permeability-increasing effect is at least partially mediated by the activation of the detergent-resistant phospholipase  $A_1$  (65). Under certain conditions, the OM becomes ruptured and permeable to macromolecules (112, 126).

The molecular mechanism by which the EDTA-treated OM allows the penetration of hydrophobic compounds is not known, but it is very reasonable to suggest that the loss of LPS will secondarily lead in the appearance of phospholipids in the outer leaflet of the OM (134, 137). The formed phospholipid bilayer patches would then act as channels through which hydrophobic compounds can diffuse (see the section on molecular mechanisms of the OM permeability increase, below).

The effect of EDTA is very weak or absent in phosphate buffer, HEPES, or nutrient media but strong in the presence of Tris (102) or other organic monovalent cations such as ethylamine, propylamine, diethanolamine, or 3-aminopropanol (223). Tris(hydroxymethyl)nitromethane, a compound lacking the primary amine group, is not able to replace Tris as a potentiating agent (102). Accordingly, a maximal OM destabilization requires not only removal of divalent cations but also replacement of those and other cations by monovalent organic amines (137).

Tris-EDTA releases approximately 30 to 50% of the LPS of smooth *E. coli* and *S. typhimurium* (102); LPS molecules with short and long O chains are released with identical efficiency (73). Even though prolonged treatment with EDTA is lethal, a very short treatment can be used to introduce macromolecules and hydrophobic compounds through the OM without affecting cell viability (102). A nonlethal short treatment with EDTA sensitizes *E. coli* to a number of hydrophobic antibiotics (erythromycin, rifampin,

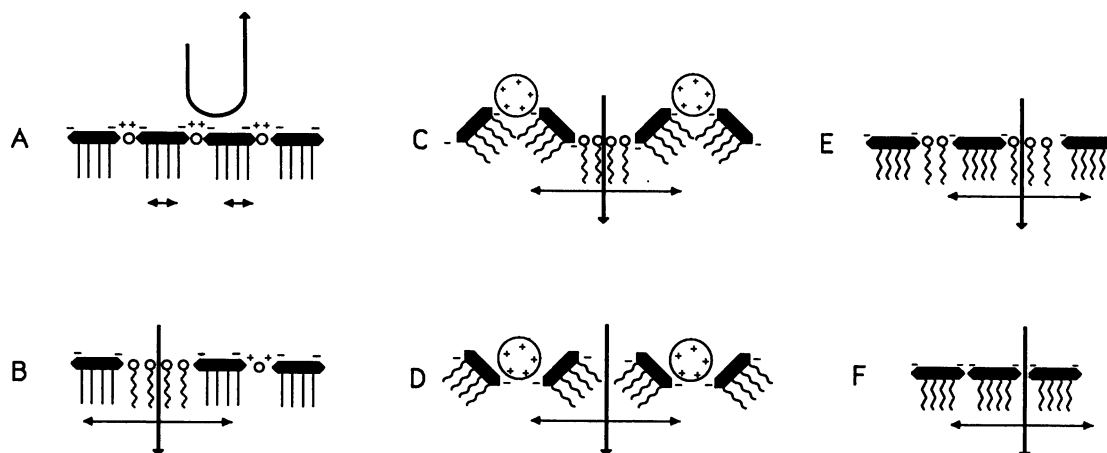


FIG. 2. Hypothetical routes of diffusion of hydrophobic solutes through the OM. For simplicity, only the outer leaflet of the OM bilayer is shown. (A) Intact OM. The anionic LPS molecules (only simplified lipid A part shown) are linked by divalent cations ( $Mg^{2+}$  or  $Ca^{2+}$ ). LPS exists in an ordered quasicrystalline arrangement (as illustrated with stiff fatty acids). Lateral diffusion (the horizontal left-and-right arrow) in the monolayer is minimal. Hydrophobic molecules (the long arrow) cannot penetrate the monolayer. (B) The OM of deep rough mutants. Hydrophobic molecules penetrate through the phospholipid domain, which has strong lateral diffusion. (C) PMBN-treated OM with phospholipids present in the outer leaflet. The supramolecular structure of LPS is less ordered (flexible fatty acids) than in intact OM. The phospholipid domain acts as a channel for hydrophobic molecules. (D) PMBN-treated OM. Hydrophobic molecules are assumed to penetrate through the LPS domain, which could have become more permeable as a result of alterations in the supramolecular structure. (E) EDTA-treated OM. Owing to removal of divalent cations, part of the LPS is lost. Phospholipids fill the void and allow hydrophobic diffusion. The remaining LPS could have disordered supramolecular structure. (F) EDTA-treated OM in which hydrophobic molecules diffuse through the disordered LPS domain. Symbols: , LPS; , phospholipid; , divalent cation; , PMBN.

novobiocin, actinomycin D, and cloxacillin) by a factor of 70 to 2,300 (166) but not at all to cephaloridine, which penetrates the OM through the porin pathway. These sensitization factors are comparable to those observed with PMBN.

The polymyxin-resistant *pmrA* mutants of *S. typhimurium*, which have an altered LPS (see below), release significantly less LPS upon treatment with Tris-EDTA than do their parents (196) and, moreover, are resistant to the Tris-EDTA-induced increase in OM permeability to lysozyme, deoxycholate, and bacitracin (196). *P. aeruginosa* strains which overexpress the cationic OM protein OprH are resistant to the bactericidal action of 10 mM EDTA and display reduced susceptibility to gentamicin but not to polymyxin (10).

Other chelators which have been shown to permeabilize the OM include nitrilotriacetate (64) and sodium hexametaphosphate (HMP) (206). Gram-negative enteric bacteria tolerate high concentrations of HMP without the loss of viability; this chelator can be used conveniently to sensitize such bacteria to hydrophobic antibiotics. Polyglutamate, polyaspartate, polygalacturonate, and phytate (all as their Na salts) are much less effective permeabilizers than HMP (206).

Acetylsalicylate and ascorbate have been shown to increase the permeability of *P. aeruginosa* OM to the hydrophobic probe nitrocefin in HEPES buffer (64). On this basis, these drugs have been regarded as permeabilizers and have been suggested to act by a mechanism involving either chelation or reduction (58–60, 64, 120). However, their effect on the uptake of another hydrophobic probe, NPN, did not significantly differ from that of the buffer control (64). Furthermore, both drugs failed to sensitize *P. aeruginosa* to hydrophobic or amphipathic antibacterial agents (deoxycholate, rifampin, fusidic acid) under conditions where EDTA and HMP were fully active (200). Accordingly, the perme-

ability-increasing effect of acetylsalicylate and ascorbate appears to be very restricted.

Also, feroxacin and other fluoroquinolones have been claimed to permeabilize the OM and to exert this function by chelating OM-bound divalent cations (24). However, published experimental evidence for this is very scarce and is based on observations made after a rather long treatment of bacterial cells with feroxacin at concentrations far above its MIC (feroxacin MIC for the *E. coli* strains used in the study,  $\leq 1 \mu\text{g/ml}$ ). Thus, it was shown that a pretreatment of *E. coli* for 1 h with feroxacin (10  $\mu\text{g/ml}$ ) increased the periplasmic hydrolysis of benzylpenicillin by 20%, compared with the hydrolysis in an untreated control (24). Furthermore, whereas the growth of *E. coli* halted approximately 20 min after the addition of feroxacin (5  $\mu\text{g/ml}$ ), bacteria slowly (after more than 40 min) started to lyse when deoxycholate was also present (24). The OM-permeabilizing effect of other fluoroquinolones was reported to be similar to that of feroxacin (24). It is possible that the effects observed are secondary and result from other, more direct effects of fluoroquinolones on the cell. In contrast to EDTA, HMP, and PMBN, the fluoroquinolone compound ofloxacin does not sensitize *P. aeruginosa* to hydrophobic antibiotics (rifampin, fusidic acid) in growth inhibition assays (205).

#### MOLECULAR MECHANISMS OF THE OM PERMEABILITY INCREASE

Intact OM of wild-type enteric bacteria prevents the diffusion of hydrophobic solutes, because its outer leaflet contains no glycerophospholipids but only highly ordered, quasicrystalline LPS (88, 134, 137). Therefore, this leaflet lacks the marked lateral diffusion typical of glycerophospholipid-containing membranes (134). On the other hand, poly-



cation- or chelator-treated hyperpermeable cells could hypothetically have phospholipids in the outer leaflet of the OM and/or could have their LPS less ordered, as a result of altered LPS-LPS interactions (Fig. 2). Accordingly, these bacteria could resemble heptoseless LPS mutants which do have phospholipids in the outer leaflet and which might have weakened LPS-LPS interactions (134, 137). Both these alterations could be thought to increase OM permeability, but in the case of deep rough mutants, it appears that the presence of phospholipids (i.e., the generation of phospholipid bilayer patches in the OM) might be the major mechanism (134) (Fig. 2).

No studies have been conducted to find whether there are phospholipid bilayers in the OM of PMBN-treated cells. One reason for this lack is obvious; the OM-impermeable probes which were used to detect phospholipids on the surface of deep rough mutants (CNBr-activated dextran, phospholipase C [80]) are far from ideal and might themselves have access through the OM, if transient cracks are present. Similarly, evidence for phospholipid bilayers in the OM of EDTA-treated cells is lacking. It would, however, be very plausible to suggest their presence, since the OM must compensate the EDTA-induced massive and instantaneous loss of LPS (134, 137). A rather convincing proof could be the demonstration that a suitable cross-linking reagent can cross-link LPS with phosphatidylethanolamine in EDTA- (or polycation-) treated cells.

The extent to which polycations alter the ordered supramolecular arrangement of LPS also remains to be elucidated. PMBN has been shown to have fluidizing (i.e., disordering) effect on lipid bilayer membranes made from rough LPS (164), but details from this study are not yet available. The fact that PMBN is able to sensitize deep rough mutants (which have phospholipid bilayers in the OM [see above]) also supports the possibility that the mechanism of PMBN-induced sensitization differs from that present in those mutants (216). Structural alterations could be studied by probing polycation-LPS complexes with such fluorescent or electron spin resonance probes which report lipid phase transitions. Cationic electron spin resonance probes such as CAT<sub>12</sub> (148), which compete with the polycations for the multiple binding sites in LPS, are not suitable for this purpose. Phase transitions could also be detected by microcalorimetry. Perhaps the most suitable methods are X-ray diffraction and Fourier transform infrared spectrometry; by both of these methods, the arrangement of lipid A has been shown to be highly ordered (17, 88, 89). Studies with various synthetic lipid A derivatives have indicated that rather small chemical alterations profoundly reduce the ordered supramolecular structure (89); thus, it could be expected that a bulky LPS-bound polycation, such as PMBN, also has such an effect. Besides the effect of polycations, that of various organic monocations including Tris should be studied, since, as discussed above, the permeabilizing effect of EDTA is dependent on such monocations.

If polycations disorder the quasicrystalline structure of LPS monolayer, this could result in greatly enhanced lateral diffusion of LPS and cause, or contribute to, the increase in the diffusion of hydrophobic solutes through the OM. To quantify the diffusion under controlled conditions, an artificial asymmetric LPS-phospholipid bilayer membrane, prepared as described by Seydel et al. (169), can be expected to be instrumental. In contrast to polymyxin B, PMBN does not increase ionic conductance (diffusion of electrolytes) through this membrane (164); it would now be very interest-

ing to test the effect of PMBN and other cations on the diffusion of a suitable hydrophobic probe.

### BACTERIAL STRAINS RESISTANT TO OM-PERMEABILIZING AGENTS

The *pmrA* mutants of *S. typhimurium* are resistant to polymyxin. They tolerate 20 to 100 times higher concentrations of polymyxin B (195, 196, 213) and bind 4 times less of it (195, 213) than their parents do. In contrast to their parents, they do not lose their OM permeability barrier to macromolecules such as lysozyme and the periplasmic  $\beta$ -lactamase or to the anionic detergent deoxycholate upon polymyxin treatment (196, 208).

The *pmrA* strains are also resistant to the effects of PMBN and EDTA (see above), as well as to the bactericidal and OM permeability-increasing actions of polylysines and protamine (196, 197). Treatment with high concentrations of cations such as Tris, Na<sup>+</sup>, and Mg<sup>2+</sup> in the cold releases a significantly smaller proportion of the periplasmic  $\beta$ -lactamase from the *pmrA* strain studied than from its parent (196). Furthermore, the *pmrA* strains show decreased binding of and sensitivity to BPI/CAP57 (45, 171, 180, 181) and azurocidin/CAP37 (171, 172, 180), the cationic leukocyte proteins described above. They are also more resistant than their parents to intraphagocytic killing in polymorphonuclear neutrophils (181).

The isolated LPS from the *pmrA* mutants binds remarkably less polymyxin than does the LPS from the parent strains (195, 212). Furthermore, although erythrocytes coated with parent-type LPS lyse upon treatment with polymyxin, those coated with the *pmrA* LPS do not lyse (20). The LPS from four independent *pmrA* mutants contains four- to sixfold-larger amounts of 4-aminoarabinose and also larger amounts of ethanolamine than does the LPS from their corresponding parent strains (212); these amino compounds esterify the phosphate groups in LPS. This makes the mutant LPS less acidic and can obviously explain the decreased binding of polymyxin. The *pmrA* mutation is located in the *S. typhimurium* chromosome at approximately 96 to 99 min (108). Unfortunately, the mutated gene has not been identified yet, and, accordingly, the gene product encoded by the *pmrA* locus is not known.

Also, polymyxin-resistant isolates of *E. coli* have been reported (114), and the LPS from two of these has received preliminary study (147). According to nuclear magnetic resonance analysis, the phosphates in their LPS are significantly more esterified than those in the parent-type LPS; hence it was suggested (147) that the resistance is due to modifications that result in less acidic LPS, as in the *pmrA* mutants of *S. typhimurium*. However, the esterifying compounds were not identified and the mutations were not mapped.

*Proteus mirabilis* and other *Proteus* species are inherently very resistant to polymyxin (182), BPI/CAP57 (9, 45, 170), and azurocidin/CAP37 (170, 172), as well as to the osmotic shock procedure (125), which involves Tris-EDTA. *Proteus* strains are also resistant to the OM permeability-increasing action of PMBN (218, 221) and bind PMBN very poorly (215). Furthermore, they are resistant to the small cationic peptides cecropins (75), L- and all-D-magainins (12, 240, 241), and bactericins Bac5 and Bac7 (51), but not to defensins (170). Whereas the MIC of gentamicin for the polymyxin-resistant mutant of *E. coli* was reported to be increased fourfold over that for the parent strain (61), *Proteus* strains are as sensitive to gentamicin as are poly-



myxin-sensitive enteric bacteria (235); also, the *pmrA* strains of *S. typhimurium* do not show increased resistance to gentamicin (196).

The mechanism of polymyxin resistance in *Proteus mirabilis* has been suggested (215) to be related to the high content of phosphate-linked 4-aminoarabinose in its LPS (177). *Proteus mirabilis* chemotype Re LPS binds significantly less polymyxin than does the analogous Re LPS from polymyxin-sensitive *S. typhimurium* (212). A mutant of *Proteus mirabilis* elaborates LPS which lacks 4-aminoarabinose and binds more polymyxin than does the LPS from the resistant control strain (78). This mutant is polymyxin sensitive. Other bacteria which inherently have a high content of phosphate-linked 4-aminoarabinose in their LPS are *Chromobacterium violaceum* (66) and *Pseudomonas cepacia* (31). Both are resistant to polymyxin (115, 119), and *P. cepacia* is also known to be resistant to EDTA (119) and HMP (206). In these bacteria, as well as in *Proteus mirabilis* and the *pmrA* mutants of *S. typhimurium*, 4-aminoarabinose esterifies the phosphate group present in the nonreducing glucosamine (i.e., GlcN-II) of lipid A (31, 66, 177, 212). *Bacteroides fragilis* is polymyxin resistant (77) and lacks this phosphate group (230). Accordingly, the presence of free, unesterified phosphate in GlcN-II is probably crucial for the effective binding of polymyxin to LPS; bacteria in which this phosphate is either lacking or substituted by cationic groups, are polymyxin resistant.

Polymyxin-resistant isolates of *P. aeruginosa* are also known (30, 57, 137). Since some of these were found to produce excessive amounts of the OM protein OprH (former designation, protein H1), it was originally proposed that the mechanism of polymyxin resistance in *P. aeruginosa* is mediated by this protein (57, 60, 127, 128). However, newer results indicate that the overexpression of OprH does not confer resistance to polymyxin (10, 159).

#### BUFFER IONS AS COMPETITORS OF THE PERMEABILIZERS

The OM-permeabilizing effect of cationic agents is inhibited by divalent cations, presumably because there is competition for the same anionic binding sites in the LPS. Many of the commonly used complex growth media contain surprisingly low concentrations of  $Mg^{2+}$  and  $Ca^{2+}$  (50 to 80  $\mu M$   $Mg^{2+}$  in tryptone-yeast extract-glucose broth [229]). Therefore, the inhibition is not necessarily manifested in those media. Aminoglycosides and Tris are completely inhibited by 1 mM  $Mg^{2+}$  (64). PMBN is effective in the presence of 1 mM  $Mg^{2+}$  (90) but loses its activity in a medium containing 10 mM  $Mg^{2+}$  (90). Also, the permeabilizing action of pentyllysine (201), longer lysine polymers (64), and BPI (231), as well as the bactericidal action of ECP (99) and bactericins Bac5 and Bac7 (179), is inhibited by divalent cations. As expected, divalent cations displace cationic probes (tritiated PMBN, dansylated polymyxin) from bacterial membranes and isolated LPS (118, 215); in this displacement,  $Ca^{2+}$  is more active than  $Mg^{2+}$  on a molar basis (215).

Also, monovalent cations have some competitive effect. The OM-permeabilizing action of aminoglycosides (61) and pentyllysine (201) is inhibited by 150 mM  $Na^+$  (i.e., by physiological ionic strength), as is the antibacterial effect of many other cationic substances such as defensins (55, 98, 167, 219), bactericins (179), azurocidin (172), ECP (99), and cathepsin G (96, 139). On the other hand, PMBN (201, 209–211, 217–219, 221), deacylpolyoxymyxin B (DAPB) (85, 220), other polymyxin derivatives (85), lysine<sub>20</sub> (210, 211),

protamine (210, 211), and BPI (231) are clearly less sensitive to inhibition by monovalent cations and are effective in physiological growth media and buffers.

The effect of EDTA and other chelators is naturally poor in growth media which contain divalent cations.

#### PROBES USED IN THE PERMEABILIZER STUDIES

The pioneering studies by Warren et al. in 1957 (226) and Repaske in 1958 (155), which demonstrated the permeabilizing action of polymyxin and EDTA, were performed by using lysozyme as a probe. Similarly pioneering was the study of Leive in 1965 (100) that EDTA sensitizes *E. coli* to actinomycin D. Since these studies, lysozyme and hydrophobic antibiotics have been widely used as probes for OM permeabilization (57, 102, 137). Assays measuring the leakage of periplasmic enzymes, the sensitization to detergent-induced lysis, the uptake of the fluorescent hydrophobic probe NPN, or the crypticity of the periplasmic  $\beta$ -lactamase have also been used (57, 137).

Ruptured OM allows lysozyme to diffuse in to exert its lytic action, as it allows periplasmic enzymes to leak out. On the other hand, being a 14.3-kDa protein, lysozyme is hardly any suitable probe to detect more subtle alterations such as the generation of phospholipid bilayer patches in the OM. Lysozyme is polycationic (pI, 10.5) but lacks any significant OM-permeabilizing activity (210). However, it binds to isolated LPS (35, 142, 143) and could thus compete with other cationic agents or facilitate its own penetration through the OM. These phenomena could interfere in the assays, particularly when polycations which have only a low affinity to LPS are being studied.

Measuring the sensitization to hydrophobic probe antibiotics is an indirect way to study OM permeabilization. On the other hand, it is also a very concrete method, especially if permeabilizers are planned to be used to increase the OM permeability to such drugs. Mechanisms other than damage to the OM are very unlikely, if an agent markedly sensitizes the test bacteria to a wide range of hydrophobic drugs, unrelated in their structure and action, and renders them as susceptible as are the strains which have a mutational defect in the OM permeability barrier (Table 3). The sensitization can be quantified as the decrease of the MIC of the probe antibiotic (Fig. 3A and B). When bactericidal probe antibiotics are used, their effect on bacterial viability can be measured (Fig. 3C). Also the target-inhibiting action of the probe antibiotic can be measured; examples include the measurement of the effect of actinomycin D on nucleic acid synthesis (100, 211) and the effect of fusidic acid on protein synthesis (211).

Bacteriolytic anionic detergents (deoxycholate, dodecyl sulfate) are also applicable probes (196, 200, 206, 208, 211). Chelators such as HMP sensitize the target cell to these detergents immediately, and the lysis occurs in a few minutes (Fig. 3D) (206). The OM-damaging effect of protamine and polylysine was first shown by using deoxycholate (196); to reduce the inactivation of these polycations by electrostatic interaction, bacteria were first treated with the polycations for a short period and pelleted and resuspended in detergent-containing buffer.

NPN is an uncharged, very hydrophobic fluorescent probe and is widely used in various OM permeabilization studies (61, 64, 104, 161, 186, 191, 194, 201). A defective or damaged OM allows NPN to partition into the hydrophobic membrane environment of the OM and cytoplasmic membrane to give a characteristic bright emission peak. The LPS released in

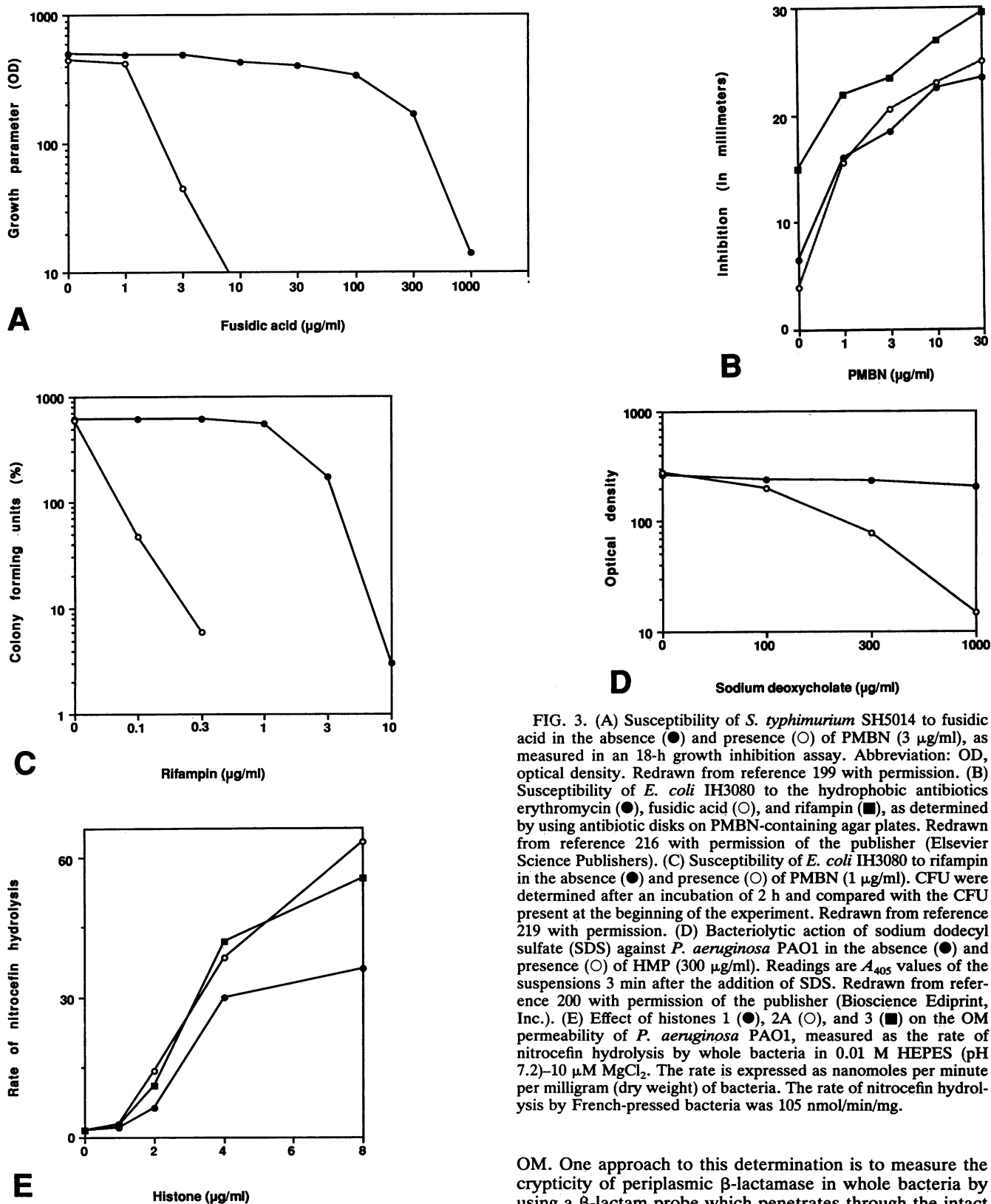


FIG. 3. (A) Susceptibility of *S. typhimurium* SH5014 to fusidic acid in the absence (●) and presence (○) of PMBN (3 µg/ml), as measured in an 18-h growth inhibition assay. Abbreviation: OD, optical density. Redrawn from reference 199 with permission. (B) Susceptibility of *E. coli* IH3080 to the hydrophobic antibiotics erythromycin (●), fusidic acid (○), and rifampin (■), as determined by using antibiotic disks on PMBN-containing agar plates. Redrawn from reference 216 with permission of the publisher (Elsevier Science Publishers). (C) Susceptibility of *E. coli* IH3080 to rifampin in the absence (●) and presence (○) of PMBN (1 µg/ml). CFU were determined after an incubation of 2 h and compared with the CFU present at the beginning of the experiment. Redrawn from reference 219 with permission. (D) Bacteriolytic action of sodium dodecyl sulfate (SDS) against *P. aeruginosa* PAO1 in the absence (●) and presence (○) of HMP (300 µg/ml). Readings are  $A_{405}$  values of the suspensions 3 min after the addition of SDS. Redrawn from reference 200 with permission of the publisher (Bioscience Ediprint, Inc.). (E) Effect of histones 1 (●), 2A (○), and 3 (■) on the OM permeability of *P. aeruginosa* PAO1, measured as the rate of nitrocefin hydrolysis by whole bacteria in 0.01 M HEPES (pH 7.2)–10 µM  $MgCl_2$ . The rate is expressed as nanomoles per minute per milligram (dry weight) of bacteria. The rate of nitrocefin hydrolysis by French-pressed bacteria was 105 nmol/min/mg.

large quantities by many of the permeabilizers (73, 102, 211) binds NPN and also allows it to emit this peak (207).

Understandably, none of the methods presented above allows the determination of permeability rates through the

OM. One approach to this determination is to measure the crypticity of periplasmic  $\beta$ -lactamase in whole bacteria by using a  $\beta$ -lactam probe which penetrates through the intact OM rather slowly but traverses through the permeabilizer-treated OM. Such probes include the chromogenic  $\beta$ -lactams nitrocefin and PADAC [7-(thienyl-2-acetamido)-3-(2-(4-*N,N*-dimethylaminophenylazo)pyridium methyl)-3-cephem-4-car-

boxylic acid]. The former is useful with *P. aeruginosa*, in which the porin-mediated permeability is exceptionally low (135), whereas the latter can be used in the study of many bacteria, since, as a very large molecule, it diffuses very poorly through the porins. This method is suitable for detecting the effect of permeabilizers (64, 94, 95, 179, 201). However, for permeability rate determinations, the method is far from ideal, since many of the permeabilizers not only allow diffusion of the  $\beta$ -lactam probe to the periplasm but also release  $\beta$ -lactamase from the periplasm (211). The permeability rate calculations must therefore be corrected for the error caused by this release. Released  $\beta$ -lactamase can be quantified by measuring the activity in cell-free supernatants, obtained by filtration (152) or centrifugation (64), but significant amounts of the  $\beta$ -lactamase may be lost during this separation, presumably by adsorption to glass and plastic, especially if a dilute buffer is used (205).

Accordingly, probes which are converted by enzymes in the cytoplasm or cytoplasmic membrane can be expected to be more useful. Recently, Plésiat and Nikaido (150) determined the OM permeability rates for hydrophobic steroid probes, oxidized in the cytoplasmic membrane, in *S. typhimurium*, and, furthermore, demonstrated that submicrogram concentrations of DAPB per milliliter increased these rates by a factor of 20 to 100.

The artificial conditions used in the permeabilizer studies can yield experimental data which have, at most, only limited value in other circumstances. Thus, by using the standard nitrocefin permeation assay, for instance, histones could be regarded as potent permeabilizers (Fig. 3E), yet this property is probably insignificant since the effect is very sensitive to increasing ionic strength (205). Furthermore, on the basis of such assays, azithromycin (a dibasic macrolide antibiotic) has been suggested to permeabilize the OM of *E. coli* and to promote its own diffusion through it (46, 59, 62). However, in contrast, OM permeabilization (e.g., by PMBN or deacylpolymyxins) is needed to sensitize *E. coli* to this antibiotic (204) (Table 2). Furthermore, mutations (such as *lpxA*, *ssc*, *SS-B*, and *rfaE*) which sensitize the cell to a large number of hydrophobic antibiotics, including erythromycin, also sensitize the cell to azithromycin and in a quantitatively identical fashion (204). Therefore, the effect of azithromycin on the OM is apparently small and insignificant.

### CONCLUDING REMARKS

All polycationic agents can be expected to bind to isolated anionic LPS. However, the affinity of this binding varies; it is high with BPI and rather high with polymyxin. Furthermore, most polycations are apparently able, at least under certain conditions, to permeabilize or traverse the OM, probably by virtue of their LPS binding. As regards the relative effectiveness of the various polycations, direct comparisons are scarce and no indisputable conclusion can be reached. However, it appears that among the cationic agents characterized in sufficient detail, BPI, polymyxin and its derivatives including DAPB and PMBN, lysine<sub>20</sub> and longer polylysines, protamine, compound 48/80, batenecins Bac5 and Bac7, and cecropin have the strongest permeabilizing activity, as estimated on a molarity and weight basis in buffers or growth media with moderate ionic strength. On the other hand, several others, including defensins, pentyllysine, and aminoglycosides, may be regarded as weak permeabilizers. The effectiveness of azurocidin, magainins, and many other cationic peptides in permeabilizing the OM still awaits evaluation.

Accordingly, it is clear that the cationicity alone is not the sole determinant required for OM-permeabilizing activity. This conclusion is also supported by the finding that polymyxin derivatives which still contain the cyclic heptapeptide ring are effective permeabilizers, whereas the polymyxinlike synthetic peptides (in which this heptapeptide is not cyclic) lack the activity. Therefore, a proper conformation, including a suitable spatial location of the positive groups, is crucial. These spatial factors could be assessed by three-dimensional ligand-receptor computer analysis.

The effect of OM-permeabilizing chelators EDTA, NTA, and HMP simply involves the removal of divalent cations that interlink adjacent LPS molecules. Even though EDTA has been an invaluable tool in numerous bacterial cell studies, the indiscriminate chelating activity reduces the value of all chelators in this and various other applications. However, chelators are instrumental, under suitable conditions, in releasing proteins from the periplasm of enteric bacteria, as are several of the polycations. This can be exploited in biotechnology.

The numerous recent discoveries of antibiotic cationic peptides may stimulate the development of more effective and less toxic OM permeabilizers. In this development, problems related to toxicity may arise. Even though a derivative lacks many or some of the toxic properties of the parent molecule, as does PMBN (see above) and cecropin-melittin hybrids (16), a notably toxic property may still be present, as is the nephrotoxicity in PMBN.

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